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DEMANDE INTERNATIONALE PUBLIEE EN VERTU DU TRAITE DE COOPERATION EN MATIERE DE BREVETS (PCT)

(51) Classification internationale des brevets 5 : C12N 15/86, A61K 48/00, C12N 15/12, 15/11 — A1

(11) Numéro de publication internationale: WO 94/24297

(43) Date de publication internationale: 27 octobre 1994 (27.10.94)

(21) Numéro de la demande internationale: PCT/FR94/00421

(22) Date de dépôt international: 15 avril 1994 (15.04.94)

(30) Données relatives à la priorité:
93/04745 22 avril 1993 (22.04.93) FR

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(81) Etats désignés: AU, CA, FI, HU, JP, NO, NZ, US, brevet européen (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Publiée
Avec rapport de recherche internationale.

(54) Title: DEFECTIVE RECOMBINANT ADENOVIRUSES FOR GENE THERAPY OF TUMOURS

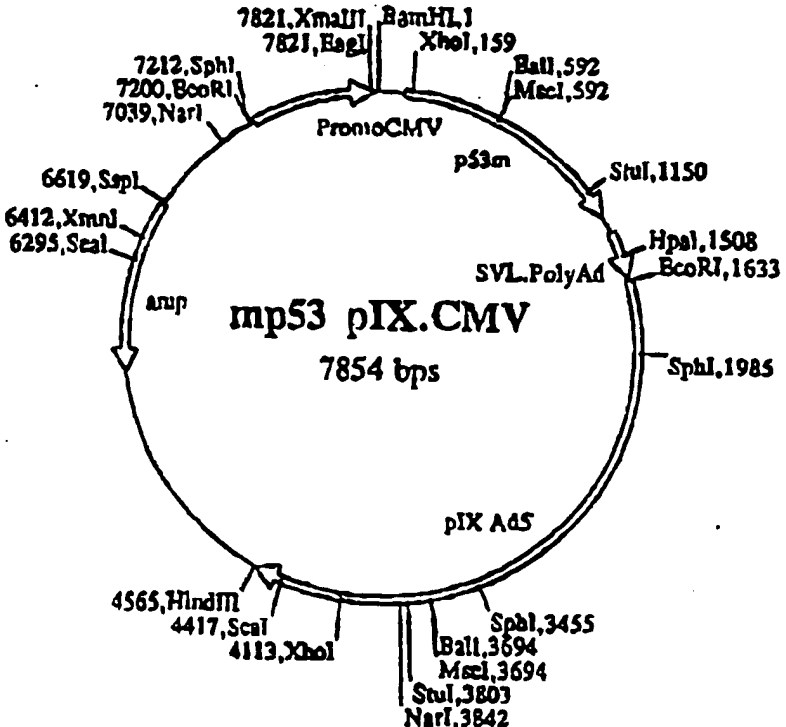
(54) Titre: ADENOVIRUS RECOMBINANTS DEFECTIFS POUR LA THERAPIE GENIQUE DES TUMEURS

(57) Abstract

Recombinant adenoviruses comprising a heterologous DNA sequence, preparation thereof, and use thereof for the treatment and/or prevention of cancer.

(57) Abrégé

La présente invention concerne des adénovirus recombinants comportant une séquence d'ADN hétérologue, leur préparation, et leur utilisation pour le traitement et/ou la prévention des cancers.



REF. AM

TRANSLATION FROM FRENCH

PCT

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INTERNATIONAL APPLICATION PUBLISHED PURSUANT TO THE PATENT COOPERATION TREATY (PCT)

(51) International patent classification[5]: C12N 15/86, A61K 48/00, C12N 15/12, 15/11	A1	(11) International disclosure No.: (43) International disclosure date:	WO 94/24297 October 27, 1994 (27.10.94)

(21) International application number: PCT/FR94/00421

(22) International application date: April 15, 1994 (15.04.94)

(30) Information regarding priority: 93/04745 April 22, 1993 (22.04.93) FR

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81) Designated contracting states: AU, CA, FI, HU, JP, NO, NZ, US; European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
With the international research report.

(54) Title: DEFECTIVE RECOMBINANT ADENOVIRUSES FOR GENE THERAPY OF TUMORS

(57) Abstract

[Recombinant adenoviruses comprising a heterologous DNA sequence, preparation thereof, and use thereof for the treatment and/or prevention of cancer.]

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DEFECTIVE RECOMBINANT ADENOVIRUSES FOR GENE THERAPY OF TUMORS

The present invention relates to recombinant vectors with a viral origin and to their use in the treatment of cancers. More specifically, it relates to recombinant adenoviruses that include a heterologous DNA [deoxyribonucleic acid] sequence whose expression in an abnormally dividing cell makes it possible, at least partially, to inhibit the division of the said cell. The invention also relates to the preparation of these vectors and to the pharmaceutical compositions that contain them.

Cell growth is regulated in an extremely subtle way by two types of signals. Some of these signals favor the multiplication of the cells, while others, conversely, cause the cells to enter into a quiescent state or cause them to become differentiated, depending on the needs of the organism. Cancers are all characterized by a disturbance in the mechanisms that control cell division, resulting in an abnormal proliferation. In most instances, therefore, the development of a cancer involves the activation of genes that favor the multiplication of cells (i.e., genes that are referred to as "proto-oncogenes", which are activated in [the form of] oncogenes), and the disappearance or inactivation of genes that inhibit cell proliferation. The present invention offers the possibility of treating cancers through gene therapy, through the administration, to tumor cells, of one or more of these genes whose expression makes it possible, at least partially, to inhibit cell proliferation.

Gene therapy consists of correcting a deficiency or abnormality (such as a mutation, an aberrant expression, etc.) through the introduction of genetic information into the affected cell or organ. This genetic information can be introduced either in vitro, into a cell that is extracted from the organ, with the modified cell then being reintroduced into the organism, or directly in vivo into the appropriate tissue. In the latter case, various techniques exist, among which are various transfection techniques that involve DNA and DEAE [diethyl-aminoethyl] dextran complexes (Pagano et al., in *J. Virol.*, Vol. 1 (1967), p. 891), DNA and nuclear proteins (Kaneda et al., in *Science*, Vol. 243 (1989), p. 375), DNA and lipids (Felgner et al., in *P.N.A.S.*, Vol. 84 (1987), p. 7413), the use of liposomes (Fraley et al., in *J. Biol. Chem.*, Vol. 255 (1980), p. 10,431), etc. More recently, the use of viruses as vectors for the transfer of genes has appeared as a promising alternative to these physical transfection techniques. In this regard, various viruses have been tested for their ability to infect certain cell populations: in particular, the retroviruses (RSV [Rous

sarcoma virus], HMS, MMS, etc.) HSV [the herpes simplex virus], the adeno-associated viruses, and the adenoviruses.

The possibility of using gene therapy to treat cancers has already been mentioned, in patent application No. WO91/15580. This application described the construction of retroviruses that contain a gene that codes for a ribozyme whose expression in a cell culture may make it possible to destroy the mRNA [messenger ribonucleic acid] of an oncogene.

The present invention is the result of the discovery that adenoviruses constitute particularly effective vectors for the transfer and expression of therapeutic genes in tumors. Specifically, adenoviruses have the advantage of not becoming integrated into the genome of the cells that they infect; of remaining there [i.e., in the cell] in a very stable manner, thereby making it possible to obtain a long-lasting therapeutic effect; and of having a very broad spectrum of hosts, which makes it possible to apply them in the treatment of cancers that affect all types of cells. Furthermore, the invention is also based on the discovery that viruses of the adenovirus type are capable of transferring and expressing genes that are capable, at least partially, of inhibiting cell division directly at the tumor level.

Therefore, a first objective of the invention consists of a defective recombinant adenovirus that contains a heterologous DNA sequence whose expression makes it possible, at least partially, to inhibit cell division.

Another objective of the invention consists of the utilization of such a defective recombinant adenovirus in the preparation of a pharmaceutical composition intended for use in the treatment or the prevention of cancers.

Within the context of the present invention, the term "defective adenovirus" refers to an adenovirus that is not capable of replicating itself autonomously in the target cell. Therefore, generally speaking, the genome of the defective adenoviruses that are utilized within the context of the present invention is stripped at least of the sequences that are necessary for the replication of the said virus in the affected cell. These regions can be either eliminated (in whole or in part), rendered non-functional, or replaced by other sequences, and particularly by the inserted gene. However, the defective virus preferably retains the sequences of its genome that are necessary for the encapsidation of the viral particles.

There are various different serotypes of adenoviruses, whose structure and properties vary somewhat. Nevertheless, these viruses are not pathogenic for humans, and particularly for non-immuno-deprived subjects. Among these serotypes, the type 2 or 5 adenoviruses (Ad 2 or Ad 5) are preferably utilized within the context of the present invention. In the case of the Ad 5 adenovirus, the sequences necessary for replication are the E1A and E1B regions.

Within the context of the present invention, the heterologous DNA sequence whose expression makes it possible, at least partially, to inhibit cell division preferably includes at least one gene selected from among tumor-suppression (or anti-oncogene) genes, or any active derivative of the said genes; the antisense genes, whose expression in the target cell makes it possible to inhibit the expression of genes that favor cell division; or genes whose expression product induces the apoptosis of the infected cell.

More specifically, the tumor-suppression genes that can be utilized within the context of the present invention include the following genes:

– The p53 gene:
The p53 gene codes for a 53 kDa [kiloDalton] nuclear protein. The mutated form of this gene, as obtained through deletion and/or mutation, is involved in the development of most human cancers (Baker et al., in *Science*, Vol. 244 (1989), p. 217). Its mutated forms are also capable of cooperating with ras oncogenes in order to transform murine fibroblasts. On the other hand, the wild-type gene that codes for native p53 inhibits the formation of transformation sites in the fibroblasts of rodents that have been transfected with various combinations of oncogenes. Recent findings emphasize that the p53 protein itself can be a transcription factor and can stimulate the expression of other tumor-suppression genes.

– The Rb gene:
The Rb gene determines the synthesis of a nuclear phosphoprotein that has approximately 927 amino acids (Friend et al., in *Nature*, Vol. 323 (1986), p. 643) and whose function is to suppress cell division by causing the cells to enter into a quiescent phase. Inactivated forms of the Rb gene have been implicated in various different tumors, and particularly in retinoblastomas or in mesenchymal cancers, such as osteocarcinomas. The reintroduction of this gene into the tumor cells where it was inactivated produces a return to the normal

state and a loss of tumorigenicity (Huang et al., in *Science*, Vol. 242 (1988), p. 1563). It was recently demonstrated that the normal Rb protein, but not its mutated forms, suppresses the expression of the c-fos oncogene (a gene that is essential to cell proliferation).

- The rap 1A gene:
The rap 1A gene (also referred to as the "k-rev1 gene") codes for a 21 kDa protein that is associated with the inner surface of the cytoplasmic membrane. At high levels, this protein is capable of causing the reversion of transformed cells that express mutated ras oncogenes (Kitayama et al., in *Cell*, Vol. 56 (1989), p. 77).

- The DCC gene:
The DCC gene codes for a protein that is homologous with the cell-adhesion proteins in the N-CAM family. This gene is very frequently deleted in colon carcinomas (Fearon et al., in *Science*, Vol. 247 (1990), p. 49).

- The k-rev2 and k-rev3 genes:
The k-rev2 gene codes for a secreted protein that has 60 amino acids, and the k-rev3 gene codes for a truncated version of a protein in the extracellular matrix. These two genes are capable of causing the reversion of NIH 3T3 cells that have been transformed by the Ki-ras oncogene.

Other genes can be utilized within the context of the present invention because of their antitumoral effect. Such genes include in particular other tumor-suppression genes that have been described in the literature, or any other gene whose expression product can induce cell apoptosis.

As indicated hereinabove, the heterologous DNA sequence can include the native tumor-suppression gene or an active derivative of the said gene. Such a derivative can be obtained through the mutation, deletion, substitution, and/or addition of one or more base pairs in the gene sequence, in accordance with conventional techniques in molecular biology. The activity of the resulting derivative can then be confirmed in vitro through tests known to those skilled in the art, such as the test described in the [following] examples.

Within the context of the invention, the heterologous DNA sequence can also include an antisense gene whose expression in the target cell makes it possible to control the expres-

sion of the genes or the transcription of the cell mRNA molecules that code for proteins that favor cell proliferation. For example, such genes may be transcribed, in the target cell, in the form of RNA molecules that are complementary to the cell mRNA molecules and that therefore block their translation into a protein.

More specifically, the antisense genes that can be utilized within the scope of the invention include all antisense sequences that make it possible to reduce the levels of production of ras, myc, fos, and c-erb B oncogenes, among others.

Generally speaking, the heterologous DNA sequence also includes promoter sequences that allow the expression of the gene or genes that are capable, at least partially, of inhibiting cell division in the target cell. The promoter sequences may be naturally responsible for the expression of the said gene when these sequences are capable of functioning in the infected cell, or else may have different origins (i.e., they may be responsible for the expression of other proteins, or may even be synthetic). In particular, a promoter sequence may promote eukaryotic or viral genes. For example, a promoter sequence may be derived from the genome of the cell that is to be infected. Similarly, a promoter sequence may be derived from the genome of a virus, including the adenovirus utilized. In this regard, examples include the promoters of the E1A, MLP, CMV [cytomegalovirus], and RSV genes, among others. These promoter sequences can also be modified through the addition of activation sequences, regulation sequences, etc. Furthermore, when the heterologous DNA sequence does not contain expression sequences, the DNA sequence can be inserted into the genome of the defective virus downstream of such a sequence. Inducible promoters can also be used.

Furthermore, in another embodiment of the invention, the heterologous DNA sequence also includes, in addition to the tumor-suppression gene or the antisense gene, a gene that codes for a specific antigen of the tumor and/or a gene that codes for a lymphokine. The effect of the combination of these genes is (i) to stop the cell division in a tumor and thus to cause the regression of the said tumor, and (ii) to increase the immune response of the organism toward the said tumor.

The specific antigens of the tumor are antigen groups that appear on the surface of tumor cells but that do not exist on the surface of non-tumor cells of the same type. Such antigens are generally utilized in the diagnosis of cancer. More recently, they have been described in connection with the implementation of anti-tumor vaccines (see European

patent No. EP 259 212). Nevertheless, they have never been combined with other therapeutic genes, as [they are] within the scope of the present invention.

More specifically, the genes that code for lymphokines include the genes that code for the interleukins (IL-1 to IL-13), the interferons, the tumor necrosis factors [TNFs], the colony-stimulating factors (G-CSF, M-CSF, GM-CSF [granulocyte colony stimulating factor, macrophage colony stimulating factor, granulocyte macrophage colony stimulating factor], etc.), and TGFβ, etc. Furthermore, the gene that codes for a lymphokine usually includes, upstream of the coding sequence, an expression sequence and a signal sequence that directs the synthesized polypeptide into the secretion pathways of the target cell. This signal sequence can be the natural signal sequence of the lymphokine, but can also consist of any other functional signal sequence or an artificial signal sequence. Such constructions make it possible in particular to increase the lymphokine levels in a very localized way, and thus, in the presence of a specific antigen of a tumor, to amplify the immune response toward a particular type of tumor, thereby providing a particularly advantageous effect. Such recombinant adenoviruses are particularly useful in the preparation of anti-tumor vaccines.

The defective recombinant adenoviruses in accordance with the invention can be prepared in accordance with any method known to those skilled in the art (Levrero et al., in *Gene*, Vol. 101 (1991), p. 195, and European patent No. EP 185 573, and Graham, in *EMBO J.*, Vol. 3 (1984), p. 2917). In particular, these adenoviruses can be prepared by means of homologous recombination between an adenovirus and a plasmid that carries, among others, the heterologous DNA sequence. The homologous recombination occurs after the co-transfection of the said adenovirus and the said plasmid in an appropriate cell line. The cell line utilized should preferably (i) be capable of being transformed by the said elements, and (ii) include the sequences that are capable of complementing the portion of the genome of the defective adenovirus, preferably in integrated form, in order to avoid the risks of recombination. The [cell] line may be, for example, the human embryonic kidney cell line 293 (Graham et al., in *J. Gen. Virol.*, Vol. 36 (1977), p. 59), which particularly contains, as an integral part of its genome, the left portion of the genome of an Ad5 adenovirus (12 percent).

The multiplied adenoviruses are then recovered and purified in accordance with the conventional methods in molecular biology, as illustrated in the examples.

The present invention also relates to a pharmaceutical composition that includes one or more defecting recombinant adenoviruses as described hereinabove. The pharmaceutical compositions in accordance with the invention preferably include a vehicle that is pharmaceutically acceptable for use in a formulation that can be injected directly into the tumors to be treated. In particular, the pharmaceutical compositions in accordance with the invention may consist of sterile isotonic solutions or dry (and in particular, lyophilized) compositions which, through the addition of sterilized water or physiological serum, as applicable, allow injectable solutions to be formed. Direct injection into the tumor to be treated is advantageous, because it allows the therapeutic effect to be concentrated in the affected tissues.

The doses of defective recombinant adenovirus that are utilized for injection can be adapted in accordance with various parameters, and particularly in accordance with the method of administration method utilized, the pathology involved, the gene to be expressed, or even the desired duration of treatment. Generally speaking, the recombinant adenoviruses in accordance with the invention are formulated and administered in the form of doses that contain between 10^4 and 10^{14} pfu per ml, and preferably from 10^6 to 10^{10} pfu per ml. The term "pfu" ("plaque forming unit") corresponds to the infectious power of a virus solution, and is determined through the infection of an appropriate cell culture and the measurement, usually after 48 hours, of the number of areas of infected cells. The methods for the determination of the pfu content of a viral solution are well documented in the literature.

Thus, the present invention offers a very effective means for the treatment or prevention of cancers. Furthermore, this treatment can also be applied to all animals, such as sheep, cattle, domestic animals (e.g., dogs, cats, etc.), horses, fish, etc., as well as to humans.

The present invention will be described more fully with the aid of the following examples, which should be construed as illustrative and not limitative.

Figure legends

[TRANSLATOR'S NOTE: No figures were included with the text submitted for translation.]

Figure 1: Representation of the mp53wtI-CMV vector

Figure 2: Representation of the mp53pIX.CMV vector

General methods in molecular biology

The methods traditionally utilized in molecular biology, such as:

- Preparatory extractions of plasmid DNA
- Centrifuging of plasmid DNA in a cesium chloride gradient
- Electrophoresis on agarose or acryamide gels
- Purification of DNA fragments by means of electro-elution
- Extraction of proteins with phenol or phenol-chloroform
- Precipitation of DNA in a saline medium by means of ethanol or isopropanol
- Transformation in *Escherichia coli*, etc.

are well known to those skilled in the art, and are abundantly described in the literature (e.g., in T. Maniatis et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), and in F.M. Ausubel et al., eds., *Current Protocols in Molecular Biology* (John Wiley & Sons, New York, 1987)).

The type pBR322 and type pUC plasmids and the phages in the M13 series are commercial products (from Bethesda Research Laboratories).

For the linkers, the DNA fragments can be separated, in accordance with their size, by means of electrophoresis on agarose or acrylamide gels, extracted with phenol or by a mixture of phenol and chloroform, precipitated with ethanol, and then incubated in the presence of the DNA ligase of the T4 phage (from Biolabs), in accordance with the supplier's recommendations.

The prominent 5' ends can be filled by means of the Klenow fragment of the *E. coli* DNA polymerase I (from Biolabs), in accordance with the supplier's recommendations. The prominent 3' ends are destroyed in the presence of the DNA ligase of the T4 phage (from Biolabs), utilized in accordance with the supplier's recommendations. The prominent 5' ends are destroyed through a treatment brought about by S1 nuclease.

Directed in vitro mutagenesis by means of synthetic oligonucleotides can be achieved in accordance with the method developed by Taylor et al. (in *Nucleic Acids Res.*, Vol. 13 (1985), pp. 8749-8764), through use of the kit distributed by Amersham.

The DNA fragments can be enzymatically amplified by means of the so-called "PCR" (polymerase-catalyzed chain reaction) method, as described by R.K. Saiki et al. (in *Science*, Vol. 230 (1985), pp. 1350-1354) and by F.A. Faloona (in *Meth. Enzym.*, Vol.

155 (1987), pp. 335-350), through the use of a so-called "DNA thermal cycler" (Perkin Elmer Cetus), in accordance with the manufacturer's specifications.

The nucleotide sequences can be verified in accordance with the method developed by Sanger et al. (in *Proc. Natl. Acad. Sci. U.S.A.*, Vol. 74 (1977), pp. 5463-5467), through use of the kit distributed by Amersham.

Examples

Example 1. Construction of the mp53wtI-CMV vector that carries the p53 gene under the control of the cytomegalovirus promoter (Figure 1).

The eukaryotic mp53wtI-CMV expression vector was constructed from the pUC91 plasmid, through the insertion of the following constituents:

– A promoter region of viral origin that corresponds to the precocious cytomegalovirus (CMV) promoter. This region in the vector is surrounded by single EcoRI-SphI restriction sites at the CMV / pUC junction, and [by single] BamHI sites at the CMV / p53 junction. The presence of single sites flanking the promoter region makes it possible to replace the CMV region with any other promoter. Thus, a second series of vectors is obtained in which the p53 gene is placed under the control of an inducible promoter, i.e., the metallo-thionine promoter, which is inducible by heavy metals (e.g., cadmium and zinc).

– An 1173 base-pair sequence that corresponds to the cDNA [i.e., complementary or copy DNA] that codes for the wild-type form of the p53 mouse protein (as described by Zakut-Houri et al., in *Nature*, Vol. 36 (1983), p. 594). In this construction, the suppressor gene is in the form of cDNA, i.e., stripped of introns. The particular feature of this construction is that it allows the size of the vector to be reduced. It has also been found that the expression levels that have been obtained are comparable regardless of the presence or absence of introns.

– A polyadenylation signal for the late or delayed SV40 virus genes, which corresponds to a very effective polyadenylation signal. Two single restriction sites (SalI and HindIII) are located downstream of the polyadenylation signal. These sites enable the insertion of the pIX regions of the adenovirus (see also Example 3 below).

Example 2. In vitro activity of the mp53wtI-CMV vector.

The functionality of the mp53wtI-CMV vector has been confirmed in vitro through [its] transitory expression in HeLa [Henrietta Lacks] cells. In this procedure, the vector was introduced into the cells by transfection and then, 40 hours later, the p53 protein was quantified through immunofluorescence and immunoprecipitation. The results obtained show that more than 50 percent of the transfected cells induce significant amounts of the p53 protein.

Example 3. Construction of the mp53pIX.CMV vector.

The plasmids used to generate (by means of homologous recombination) the recombinant adenoviruses that express the p53 gene were constructed in the following way:

The eukaryotic mp53pIX.CMV vector was constructed through the insertion of the pIX sequence obtained from the genome of the adenovirus between the SalI and EcoRI sites in the mp53wtI-CMV [adenovirus]. The pIX sequence was isolated from the pLTR-βgal pIX recombinant plasmid (as described by Stratford, Perricaudet et al. in *J. Clin. Invest.*, Vol. 90 (1992), p. 626), by digestion by means of EcoRV and HindIII enzymes.

The resulting mp53pIX.CMV expression vector (see Figure 2) has a single HindIII site downstream of the pIX insertion thereby enabling a linearization of the construction (see also Example 4 below).

Example 4. Construction of a defective recombinant adenovirus that carries the p53 gene under the control of the CMV promoter.

The mp53pIX.CMV vector is linearized and co-transfected with a deficient adenoviral vector in the helper cells (cell line 293) that provide, in *trans* mode, the functions coded by the E1 (E1A and E1B) regions of the adenovirus.

The Ad.p53 adenovirus is obtained through homologous recombination in vivo between the mutant Ad-d1324 adenovirus (as described by Thimmappaya et al., in *Cell*, Vol. 31 (1982), p. 543) and the mp53pIX.CMV vector, in accordance with the following protocol: after linearization with the HindIII enzyme, the mp53pIX.CMV plasmid and the d1324 adenovirus are co-transfected in cell line 293 in the presence of calcium phosphate in order to allow the homologous recombination. The recombinant adenoviruses generated in this way are selected through purification on a plate. After isolation, the DNA in the recombinant adenovirus is amplified in cell line 293, thereby leading to a culture supernatant that contains the unpurified defective adenovirus with a pfu content of approximately 10^{10} pfu per ml.

The viral particles are then purified through centrifuging on a cesium chloride gradient in accordance with known techniques (in particular, see Graham et al., in *Virology*, Vol. 52 (1973), p. 456). The Ad.p53 gene can be stored at minus 80° C in 20 percent glycerol.

The ability of the Ad-p53 gene to infect cells in a culture medium and to express, in the culture medium, a biologically active form of the wild type of the p53 gene has been shown by the infection of human cells in cell line 293. The presence of the p53 gene in the culture supernatant was then demonstrated by means of a specific monoclonal antibody to the p53 gene.

This research makes it possible to demonstrate that the adenovirus does indeed express a biologically active form of the p53 gene.

CLAIMS

1. Defective recombinant adenovirus that contains a heterologous DNA sequence whose expression in a target cell makes it possible, at least partially, to inhibit cell division.

2. Adenovirus in accordance with Claim 1, characterized by the fact that it is stripped of the regions of its genome that are necessary for its replication in the target cell.

3. Adenovirus in accordance with Claim 2, characterized by the fact that it is a type Ad 5 adenovirus.

4. Adenovirus in accordance with any one of claims 1 to 3, characterized by the fact that the heterologous DNA sequence contains at least one tumor-suppression gene or an active derivative of such a gene.

5. Adenovirus in accordance with Claim 4, characterized by the fact that the tumor-suppression gene is selected from among the following genes: p53, Rb, rap 1A, DCC k-rev2, and k-rev3, or an active derivative of these genes.

6. Adenovirus in accordance with any one of claims 1 to 3, characterized by the fact that the heterologous DNA sequence contains at least one antisense [i.e., anticoding] gene whose expression in the target cell makes it possible to control the transcription or translation of genes that favor cell proliferation.

7. Adenovirus in accordance with Claim 6, characterized by the fact that the antisense gene makes it possible to reduce the levels of translation of the ras, myc, fos, and/or c-erb oncogenes.

8. Adenovirus in accordance with any one of claims 1 to 3, characterized by the fact that the heterologous DNA sequence contains at least one gene whose expression product induces the apoptosis of the infected cell.

9. Adenovirus in accordance with any one of claims 1 to 8, characterized by the fact that the heterologous DNA sequence contains promoter sequences that allow the expression, in the infected cell, of the gene that at least partially inhibits cell division.

10. Adenovirus in accordance with any one of claims 1 to 9, characterized by the fact that the heterologous DNA sequence also contains a gene that codes for a specific antigen of the tumor and/or a gene that codes for a lymphokine.

11. Utilization of an adenovirus in accordance with any one of claims 1 to 10 in the preparation of a pharmaceutical composition intended for the treatment and/or prevention of cancers.

12. Utilization in accordance with Claim 11 in the preparation of a pharmaceutical composition intended for administration directly into the tumor to be treated.

13. Utilization in accordance with Claim 11 in the preparation of an antitumoral vaccine.

14. Pharmaceutical composition including one or more defective recombinant adenoviruses in accordance with any one of claims 1 to 10.

15. Pharmaceutical composition in accordance with Claim 13, characterized by the fact that the composition is in injectable form.

16. Pharmaceutical composition in accordance with Claim 13, characterized by the fact that the composition includes between 10^4 and 10^{14} pfu per ml, and preferably from 10^6 to 10^{10} pfu per ml, of defective recombinant adenoviruses.